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INTRODUCTION

Vascularized composite allografts (VCA) are transplants containing multiple tissue types (including bone, muscle, skin, nerves and blood vessels), which offer patients restoration of function and form following severe, disabling and disfiguring injury or tissue loss, in circumstances where the results of conventional reconstructive surgery remain unsatisfactory. The high incidence of episodes of skin-targeted acute rejection, and the morbidity associated with current immunosuppression regimens, necessary throughout the life of the recipient to prevent rejection, remain significant areas in which improvement would enhance quality of life, improve the risk-benefit ratio and ultimately expand availability of these procedures to severely injured service men and women, and civilians victims of disabling and disfiguring trauma or disease (Leonard et al, 2013). The objective of the VCA laboratory at the TBRC is to develop a clinically-applicable strategy for the induction of immune tolerance of VCAs. The aim of the work supported by this award is to introduce and optimize a protocol for VCA tolerance based on the principal of delayed induction of mixed chimerism in a non-human primate model. This approach, in contrast to protocols which have already reached clinical trial for kidney transplantation, permits induction of tolerance in the context of transplantation from deceased donors – a prerequisite for clinical application in VCA. Successful induction of tolerance of VCAs using this protocol in non-human primates can be expected to lead to rapid translation to clinical trial.

KEYWORDS

Vascularized composite allograft, vascularized composite allotransplantation, restorative transplantation, transplant tolerance, mixed chimerism, delayed induction of transplant tolerance, non-human primate model.

OVERALL PROJECT SUMMARY

i. Progress against Current Objectives

Year 1: Aim 1: To Optimize the Delayed Tolerance Induction Protocol for Vascularized Composite Allotransplantation in a Non-Human Primate Model.

Task	Subtask	Months	Progress
(1.1) TASK 1. Optimize standard immunosuppression (SIS) protocol for upper extremity transplantation in nonhuman primates. (Months 0-12)	(1.1.1) SUBTASK 1. IACUC and ACURO review and approval. (Month 0-3)	Month 0-3	100%
	(1.1.2) SUBTASK 2. Orthotopic upper extremity transplants on SIS protocol (n=3). (Month 3-6)	Month 3-6	Deferred/Award modification requested (See note)
	(1.1.3) SUBTASK 3. Investigate VCA survival, frequency of complications, withdraw SIS at 6 months, document rejection process clinically and histologically. (Month 3-12)	Month 3-12	Deferred/Award modification requested (See note)
	(1.1.4) SUBTASK 4. Summarize optimal immunosuppressive requirements for VCA survival in NHPs. Analyze and summarize data on VCA rejection. Year 1 report. (Month 12)	Month 12	Deferred/Award modification requested (See note)

(1.2) TASK 2. Investigate delayed tolerance induction protocol (DTIP) for upper extremity transplantation in nonhuman primates. (Months 6-18)	(1.2.1) SUBTASK 1. Orthotopic upper extremity transplants on 4 months SIS (n=4). (Months 6-9)	Month 6-9	75%
	(1.2.2) SUBTASK 2. Delayed tolerance induction protocol, wean immunosuppression. (Months 10-13)	Month 10-13	75%
	(1.2.3) SUBTASK 3. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 10-18)	Month 10-18	75%
	(1.2.4) SUBTASK 4. Summarize preliminary data/progress on DTIP transplants for inclusion in year 1 report(Month 12)	Month 12	75%
(1.3) TASK 3. Produce LFA-3-IgG for use in Aim 2/Aim 3 (Months 0-24)		Month 0-24	50%

Table 1. Progress against objectives

Notes:

1. Task 1 Progress/Award Modification Request:

Task 1 Subtasks 2-4 have been deferred and an award modification request submitted to the GOR. These subtasks collectively address characterization of the rejection of vascularized composite allografts in non-human primate models. Data from the experiments described in these subtasks would not address a primary aim of this award, but was deemed necessary to serve as negative control data and facilitate subsequent tasks. However, during the interval between submission of our grant application, completion of the award process and ultimately completion of Task 1 Subtask 1 (IACUC and ACURO approval) data fulfilling this requirement became available from our own preliminary studies performed leveraging intramural funds, and from the work of other investigators. Therefore, completion of subtasks 2-4 would represent unnecessary repetition, and it was decided to progress directly to Task 2 Subtask 1. Our award modification request and modified statement of work are included as appendices 1 and 2 to this report.

ii. Results

Establishment of screening protocol to select optimal donor-recipient pairs for transplantation experiments

Selection of appropriate recipient and donor animals is critical for both successful performance of the experiments supported by this award, and to maximize the quantity and quality of data collected from these experiments with regard to the biology of VCAs and the mechanisms active in their acceptance and/or rejection. All experiments are performed using Mauritian origin cynomolgus macaques. We select male animals, with body weight greater than 5 kg, and no history or evidence of limb injuries. This pool of animals undergoes

blood sampling at the vendor facility, and samples are sent to our laboratory for analysis of ABO blood type, and expression of the H38 HLA Class I antigen, which we utilize as a marker of donor, to permit flow cytometric analysis of donor origin cells during mixed chimerism experiments. A further sample is sent to the national primate research center in Wisconsin, for molecular analysis of MHC genetics to provide comprehensive MHC typing data.

Following these analyses, donor-recipient pairs are selected based on ABO compatibility, H38 mismatch and full MHC mismatch in that priority order. ABO compatibility is obligatory to avoid confounding effects on VCA outcome. If fully MHC mismatched pairs are not available, selection of pairs with a degree of partial matching at either class I or class II is acceptable, as this remains consistent with clinical experience in VCA to date. This year 2 rounds of screening were performed, and we envisage 2-3 rounds per annum for the remainder of this award. Our screening and selection algorithm (A), and representative H38 (B) and MHC (C) data are presented in Figure 1 below.

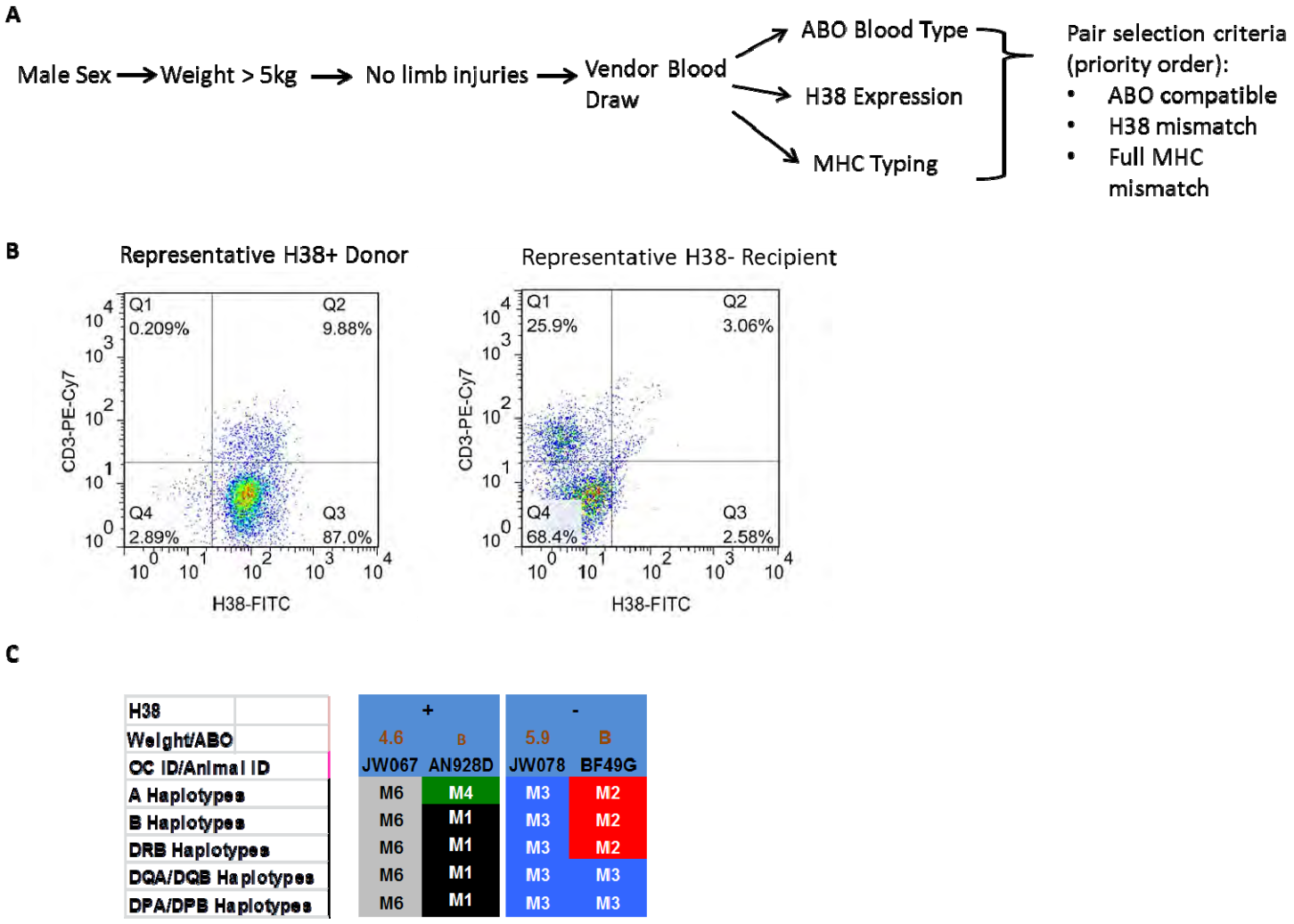

Figure 1. Screening protocol and selection algorithm for donor-recipient pairs of cynomolgus macaques. *A) Algorithm. B) Representative flow cytometry data demonstrating H38 expression by candidate donor animals, and lack of expression by candidate recipients. C) Representative MHC typing data demonstrating a pair of animals fully mismatched at MHC class I (A, B haplotypes) and class II (DRB, DQA/DQB, DPA/DPB haplotypes).*

Investigation of delayed tolerance induction protocol for upper extremity transplantation in non-human primates

Having gained all necessary IACUC and ACURO approvals, established screening and selection algorithm for recipient and donor animals, work commenced *in vivo* toward the investigation of delayed tolerance induction. Three transplants have been performed during this first annual reporting period, representing 75% of the animals currently approved for Aim 1 Task 2, which is scheduled for activity through month 18 of the statement of work. Each experiment will be described briefly in turn, identified by recipient animal identification number, with emphasis on the *in vivo* results.

M4213

Transplantation was uncomplicated. Osteosynthesis was achieved with excellent intraoperative position and fixation. Anastomoses of the radial artery and cephalic vein were immediately patent following removal of vascular clamps, and the hand reperfused rapidly. The immediate post-operative appearance of the hand is shown in Figure 2 (A-C) below, note the presence of bright-red blood at the index finger tip in (C) which was elicited by needle stick, and demonstrates perfusion of the hand.

Post-operative recovery went well, with no immediate complications noted. The hand remained well perfused on post-operative day (POD) 1, when radiographs confirmed good opposition of the radius and ulna, with no significant angulation (Figure 2 D, E).

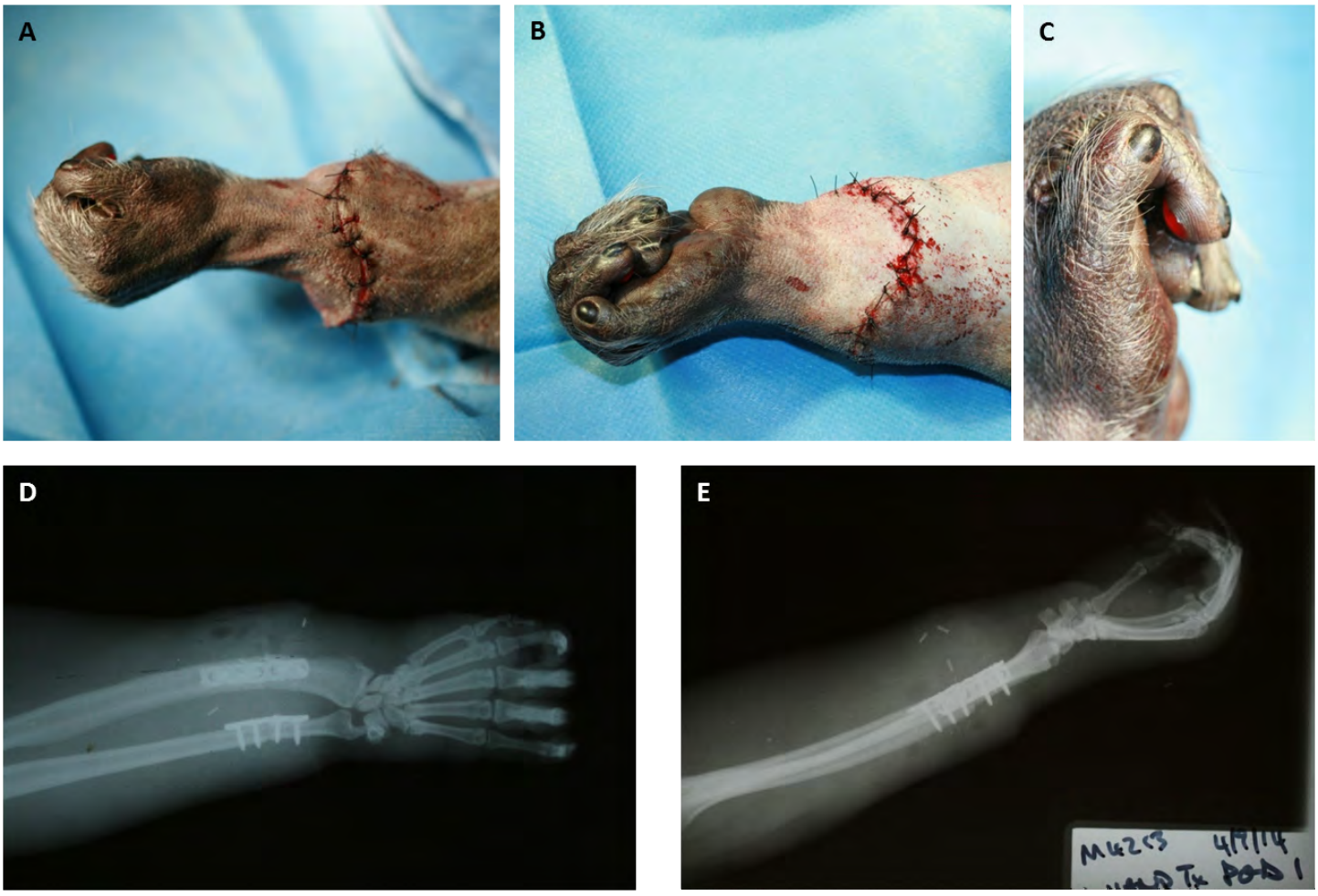

Figure 2. Immediate post-transplant appearance of transplanted hand (representative images recipient M4213). *A) Radial view, B)Volar view, C)Finger bleeding perfusion test, D) AP radiograph, E) Lateral radiograph.*

On POD2 marked bruising of the volar wrist skin of the transplanted hand was noted, along with the onset of edema in the hand, which remained well perfused. Over the remainder of the first post-operative week the animal continued to make a satisfactory recovery from surgery, and the hand remained well perfused. The edema noted on POD2 gradually resolved. Some superficial scaling of the volar wrist skin was noted, and this separated by POD9 to reveal healthy underlying skin with no evidence of persisting inflammation. Immunosuppression levels remained within target range throughout this period, suggesting that acute rejection was unlikely, and it was concluded that this scaling was consistent with ischemic insult to the volar wrist skin flaps during the immediate post-transplant period.

On POD14 increased swelling at the surgical site was noted, associated with cellulitis tracking proximally in the recipient forearm and purulent discharge from the wound (Figure 3). A modest leukopenia (21 k/ul) was noted. The animal remained afebrile. The collection was drained, samples sent for culture and antibiotic sensitivity screening and empirical antibiotic therapy started. This treatment resulted in rapid improvement, with relief of cellulitis, resolution of discharge with no further collection, and resolution of leukopenia over the next 7 days

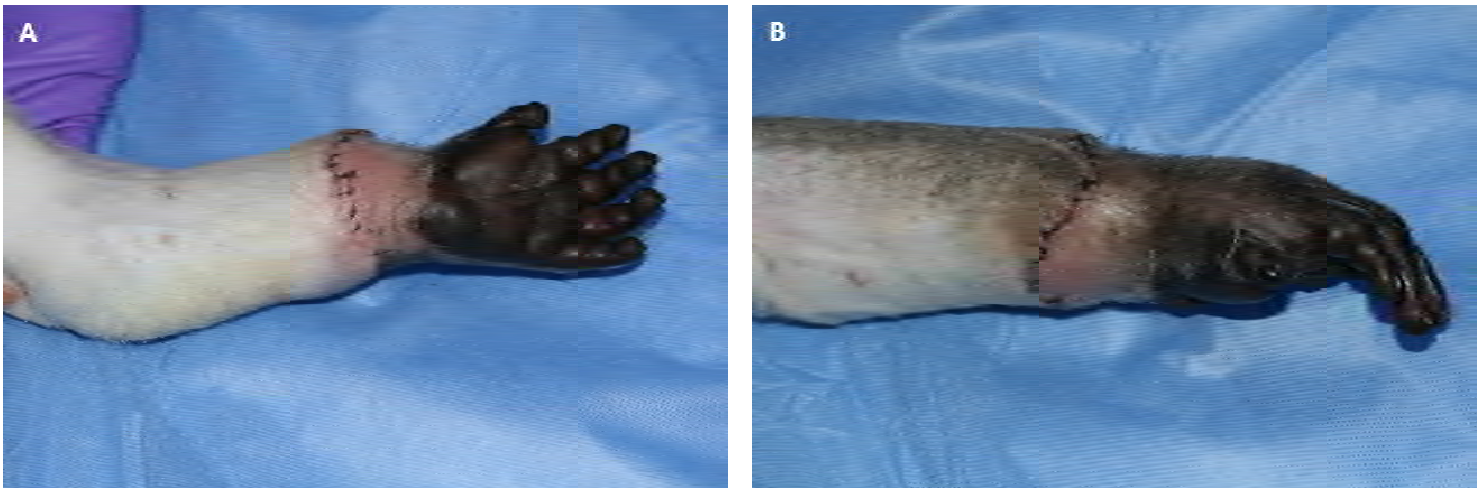

Figure 3. Appearance of M4213 upper extremity transplant on post-operative day 14. *A) Volar view B) Radial view. Note erythema tracking across skin incision into recipient forearm, and swelling along ulnar border of distal recipient forearm in A.*

Clinical status remained stable from POD21 to 28, however at the time of the scheduled protocol biopsy on POD 30 erythema restricted to the transplanted hand was noted. Indeed, the biopsy was reported as grade 1 acute rejection on the Banff scale. Erythema progressed rapidly to involve the entire hand, with induration of the skin also noted, and clear demarcation at the donor-recipient margin. Furthermore, new onset thrombocytopenia was detected with a fall from 463 k/μl to 87 k/μl over 4 days (thrombocytopenia has not previously been reported with VCA rejection but may be associated with acute rejection in renal transplantation). Per protocol, samples were collected for analysis of skin infiltrating leukocyte populations, peripheral blood T cell levels and anti-donor antibody production, and rescue therapy initiated with steroid bolus (methylprednisolone 40mg IV, to be followed by taper).

Unfortunately despite continued rescue therapy, skin rejection progressed and could not be reversed, necessitating removal of this animal from the study at POD51 with the primary end point of acute rejection prior to tolerance induction protocol.

M4313

Transplantation proceeded smoothly until the point of microvascular anastomoses, at which point the radial artery anastomosis, although demonstrably patent initially, was spasmodic and prone to poor flow despite intraoperative use of 1% lidocaine as an anti-spasmodic. Anastomosis of the cephalic vein was straightforward, but outflow was minimal. Intraoperative findings were consistent with inadequate inflow. Despite systemic heparinization, aggressive fluid therapy to maintain systolic blood pressure above 90 mmHg, and use of both blown-air heater and warm fluids, perfusion remained inadequate. A second arterial branch was anastomosed without significant benefit. In the absence of a surgically correctable inflow or outflow problem the decision was taken to complete the transplant procedure and recover the animal to a heated cage with the aim of optimizing circulation.

On POD1 slight bleeding was noted from the donor wrist skin, indicating some perfusion, however the hand remained cool and no bleeding was elicited from the finger tips. Unfortunately, despite continued anticoagulation, fluid therapy, warming and analgesia perfusion was not restored, and ischemic rubor, followed by progressive necrosis of the digits was noted and the animal was removed from study on POD7 as a primary technical failure.

M4413

Transplantation proceed without complication, with the cephalic vein and two arteries (both appearing to be branches of the radial system) patent at conclusion. Arterial pressure remained stable and satisfactory throughout.

The initial post-transplant appearance in this animal was encouraging, however, by POD2 the hand appeared cool with reduced bleeding, consistent with impaired arterial inflow. There was no evidence of bleeding or swelling at the wrist to suggest compression. Despite aggressive anticoagulation and supportive therapy, and despite this animal demonstrating excellent general recovery from surgery with good overall health status, evidence of ischemic progressed necessitating removal from the study on POD7 as a primary technical failure.

The early onset of rejection in M4213, while disappointing, was not unexpected and represents an interpretable and valuable result for our studies. The failure to achieve adequate reperfusion of the transplanted extremities in M4313 and M4413 were extremely disappointing, and while we considered such a result a real possibility we could not predict such a high incidence of technical complications in one group. This is discussed further in section iv: changes and problems and v: discussion.

Collection, analysis and cryopreservation of donor bone marrow for delayed induction of transplant tolerance

The key experimental objectives of this project are dependent on the transplantation of donor bone marrow for induction of mixed chimerism and specific tolerance of donor antigens. For each transplant performed, following procurement of the donor hand, the donor animal was euthanized by exsanguination under deep anesthesia and vertebral bodies and long bones collected for isolation of bone marrow. Following mechanical digestion and suspension of bone marrow cells into isolation buffer, samples were analysed to determine the cellular composition of the bone marrow product prior to resuspension in cryoprotective media and cryopreservation at -80^{o}C.

While the graft is collected under relatively non-sanguinous conditions, it does contain a measured content of CD3+ T cells, in which both CD4+ and CD8+ populations can be detected (Figure 4 A). As expected, large populations of myeloid antigen presenting cells were detected ($SSC^{hi}CD11b^{+}$), as were small populations of B cells (SSC^{lo} CD20+ $CD11b^{-}$) (Figure 4B) and NK cells (Figure 4 C). These populations all uniformly expressed the donor marker H38.

In subsequent experiments where long-term rejection-free survival is maintained and the tolerance induction protocol initiated 4 months VCA post-transplant, cryopreserved donor bone marrow will be thawed, samples taken to repeat this subset analysis and to assess viability, and bone marrow transplantation (BMT) performed. Chimerism analysis of recipient peripheral blood samples post BMT will include comparison of detectable donor populations to those identified within the donor BMT graft.

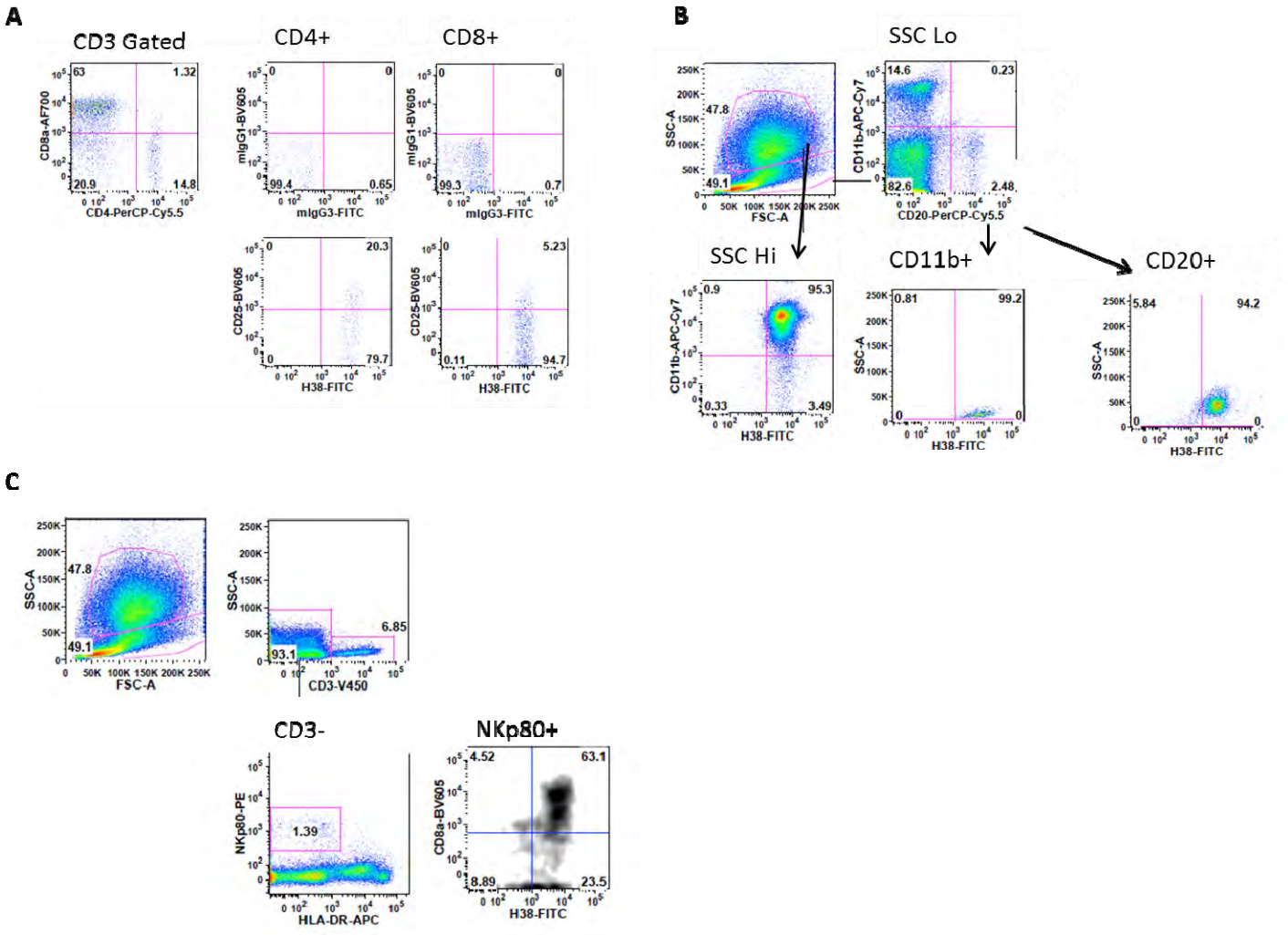

Figure 4. Analysis of donor bone marrow prior to cryopreservation (representative data). *Subset and H38 expression of A) T cells, B) myeloid cells and B cells, C) NK cells.*

Optimization of techniques for flow cytometric analysis of cutaneous leukocytes and correlation of flow cytometric data with gross and histological evidence of rejection

We have previously developed techniques for isolation and detailed flow cytometric analysis of immune system components (dermal and epidermal T cells, and epidermal Langerhans' cells) resident in or infiltrating the skin in our porcine VCA models (Leonard, manuscript in preparation). Similar protocols have been utilized for comprehensive studies of the human skin-resident immune system (Clark et al, 2006, 2007, 2012). We have now optimized these techniques for this non-human primate model system, achieving reliable isolation of populations for analysis from small quantities of biopsy material available in this model. This analysis includes subset analysis of dermal T cells, epidermal T cells and epidermal Langerhans' cells, including determination of donor- or recipient-origin in each case.

The early onset of acute cutaneous rejection in M4213 yielded the opportunity for us to apply these techniques to characterize the cutaneous immune system in this model during acute rejection. At the time of the POD30 protocol biopsy, generalized erythema was noted on gross inspection (Figure 5 A, B).

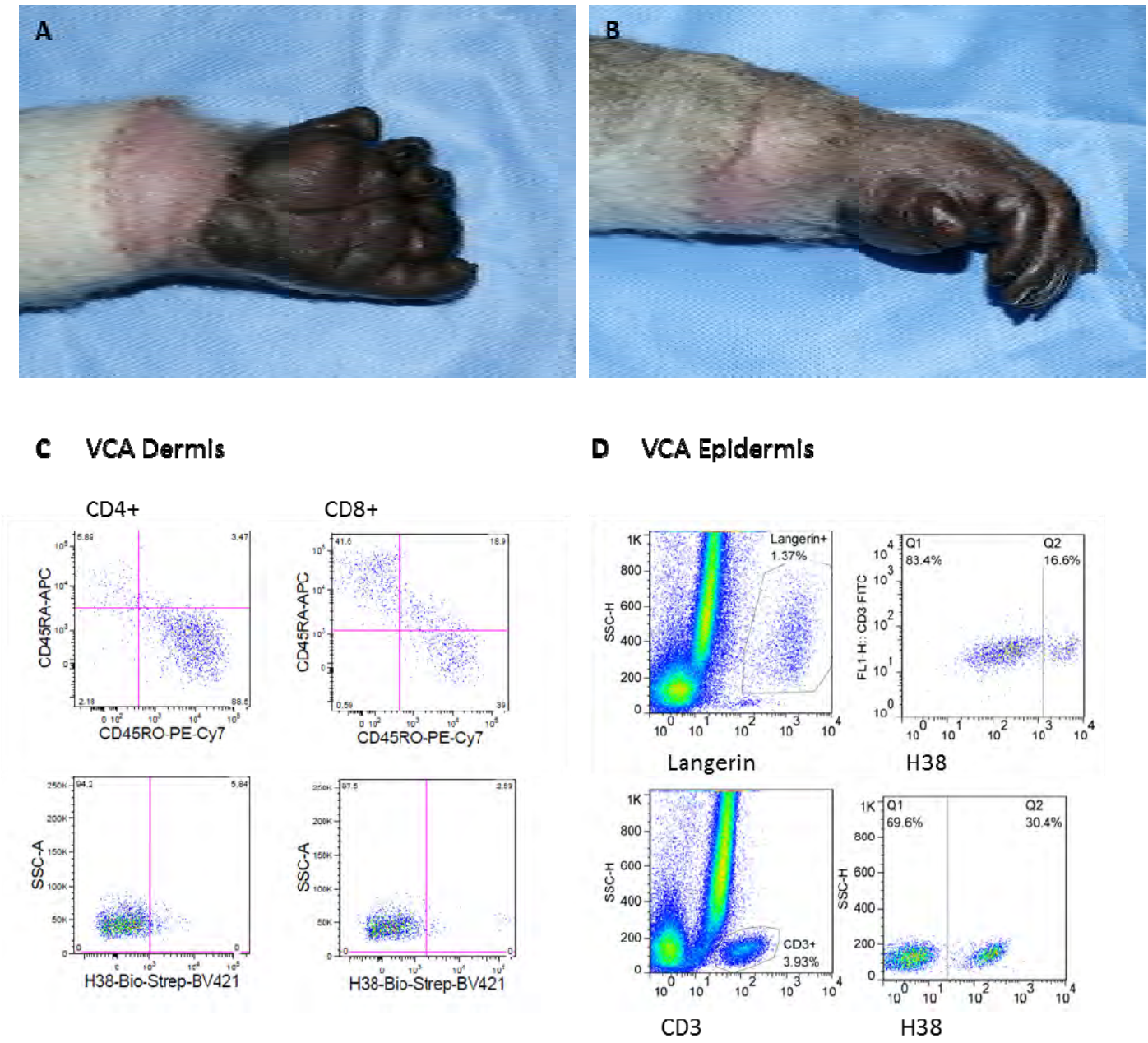

Figure 5. Flow cytometric analysis of skin leukocyte populations correlates with onset of acute rejection. *A) Gross appearance M4213 POD30 volar view. B) Radial view. C) T cell subset and chimerism analysis VCA dermis POD30 (all CD3+ gated, top panel CD45RA vs CD45RO for CD4+ and CD8+ populations, bottom panels, H38 expression for CD4+ and CD8+ populations). D) Langerhans' cell (top panels) and CD3+ T cell (bottom panels) origin in VCA epidermis M4213 POD30.*

Hematoxylin and eosin stained biopsy sections were reviewed by a board certified pathologist and scored Banff grade 1 acute rejection. Biopsy samples for flow cytometric analysis were digested in dispase II to facilitate separation of dermis and epidermis, which were then subjected to further digestion in collagenase D and trypsin respectively to release resident leukocytes, and single cell suspensions prepared. These suspensions were then stained with antibodies to CD3, CD4, CD8, CD45RA, CD45RO and H38 (dermis) and CD3, Langerin and H38 (epidermis) for detection by flow cytometer.

As demonstrated in Figure 5 C, both CD4+ and CD8+ T cells were identified, the former predominantly displaying the $CD45RA^{-}CD45RO^{+}$ phenotype consistent with memory T cells, while the CD8+ population demonstrated a more even distribution of memory and naïve phenotypes. The vast majority of T cells in VCA dermis at this point were of recipient origin ($H38^{-}$) consistent with infiltration of recipient T cells and loss/rejection of donor-origin cells.

In epidermis (Figure 5 D), recipient origin CD3+ T cells were also identified, as were recipient Langerhans' cells. Interestingly, in both cases, donor-origin cells persisted at this time point.

iii. Changes/Problems

Award Modification Request

We have submitted an award modification request seeking approval indefinitely defer Task 1 Subtasks 2-4 and redistribute the experimental animals and resources allocated for this work to the remainder of the approved work. This request originated early in this reporting period when it became clear that data addressing the experimental aims of these subtasks had become available from other sources, thereby rendering these initial experiments repetitive.

While this request was prepared and addressed the decision was taken to temporarily defer this work and, following approval of the proposed work by IACUC and ACURO (Task 1 Subtask 1) proceed to Task 2 in order to maintain progress towards our overall goals and against the statement of work. As reported above, we encountered technical challenges in two animals necessitating their early removal from study. This represents a significantly higher rate of technical failure/unrecoverable complication than encountered during our preliminary studies in this model. We do not anticipate the rate of unrecoverable complications to be sustained at this level, but nonetheless, permission to redistribute the animals allocated to Task 1 Subtasks 2-4 to the remaining groups in this project, effectively increasing the number of available animals by n=1 for each remaining group, would be beneficial in terms of maximizing confidence in the resulting data.

Technical Failures

Given the technically challenging nature of this model, technical failures resulting in removal of some animals from protocol in the early post-transplant period are not surprising, indeed, they are to be expected. However, loss of two animals in succession from one group with an intended size of n=4 recipients, represents a higher rate of such complications than predicted on the basis of our preliminary studies using this model.

In both cases, M4313 and M4413, the cause of failure appeared to be primary failure of arterial inflow to the transplanted hand; this in itself is quite surprising as microsurgical complications most commonly arise from venous congestion as a result of kinking or thrombosis in the low pressure venous system. Again in both cases, arterial insufficiency developed despite patent arterial anastomoses and demonstrable arterial flow intraoperatively.

A thorough analysis of both cases was performed, and while no key error or factor could be identified as the root cause of failure in either case one potential error was identified for M4313, and a number of potential factors identified where minor optimizations might be helpful in reducing the risk of technical failure.

In M4313 it was noted during retrospective analysis that the technique used for flushing the donor hand with heparin saline and perfusion solution was slightly modified from previous experiments, in that a smaller volume syringe was used and higher infusion pressure developed. While it was not clear that excessive pressure was used, it cannot be excluded that sufficient pressure was produced in the system to result in barotrauma to the capillary bed. Significant damage to the capillary bed would be expected to negatively effect tissue perfusion immediately following removal of clamps, and to promote thrombosis and graft failure thereafter. Corrective action for this potential error is to ensure low-pressure flushing in all cases.

A number of more general areas offering opportunities for minor optimization with the aim of reducing the risk of early technical failure have also been identified. None of these represent an area of deficiency in the original protocol, but rather minor adjustments which can be expected to have a beneficial cumulative effect.

1. Enhanced intravenous fluid support: maintenance of optimal intravascular volume can contribute to improved outcomes following microvascular surgery. This can be challenging to achieve in non-human primates, where indwelling vascular lines may be subject to damage or removal by the animal, or serve as a conduit for infection. Equally, continual reliance on percutaneous venous access for administration of IV fluids and collection of blood samples can have a deleterious effect on peripheral veins, rendering access increasingly difficult. We have therefore submitted an amendment to IACUC and ACURO for approval to place an indwelling vascular access port (VAP) in each subsequent recipient animal. These ports are accesses percutaneously using aseptic technique and a Huber needle and provide reliable access to the central venous system without open surgical intervention or repeated peripheral vascular access.

2. Enhanced monitoring of the transplanted part: Patients undergoing microsurgical replantation of amputated tissue, reconstructive free-tissue transfer or restorative transplantation will experience very high frequency monitoring of the tissue in question by experienced clinical staff during the early post-operative period. This may be augmented by technological monitoring such as temperature, pulse oximetry or laser Doppler, but frequent clinical assessment is a mainstay. One of the most significant challenges of this, or other, VCA models in non-human primates is the inaccessibility of the transplant for assessment without a brief period of anesthesia. We are actively exploring options for remote telemetric monitoring of extremity transplants in our model. Until such time as a reliable system which accurately detects declining perfusion is available we will continue to rely on careful clinical assessment at the maximum frequency permitted by our IACUC/ACURO approved protocols, and a maintain a low threshold for intervention to improve perfusion or, where applicable, return to the operating room for surgical correction.

3. Modification of transplant technique: While we have achieved successful transplants using our current surgical technique, we recognize that modifications may be possible to optimize conditions for success. At present, all vascular anastomoses, neurorrhaphies, tenorrhaphies and osteosynetheses are performed in a narrow zone approximately 3-5cm proximal to the wrist. This technique minimizes longitudinal dissection in the recipient, potentially minimizing trauma proximal in the recipient forearm, and also minimizes the length of the donor skin flaps, thus minimizing the risk of ischemic complication in these flaps. However, placement of the vascular anastomoses in this zone, overlying multiple tenorrhaphies and immediately under the interdigitating skin flaps does expose them to injury during wound closure, compression and potential prothrombotic factors in this zone of considerable tissue injury and healing. We have previously described the volar forearm fasciocutaneous extension as a strategy to improve vascular reliability in clinical hand transplantation (Eberlin et al, 2014). Application of this paradigm to this model might facilitate greater reliability by moving the arterial anastomosis proximal, in conjunction with the volar fasciocutaneous extension, to larger caliber vessels. Indeed, post mortem anatomic examination of the arm and ischemic allograft in M4313 and M4413 (Figure 6 A, B) revealed thrombosis extending distal from the level of the arterial anastomoses in the zone of maximum tissue disruption (Figure 6 C). Bifurcation of the radial artery was identified 1-2cm proximal to this zone, above which vessel diameter was noticeably larger (Figure 6 D). In contrast, the ulnar artery, another potential source of arterial inflow was found to be vestigial in this (Figure 6 E) and the majority of other animals examined.

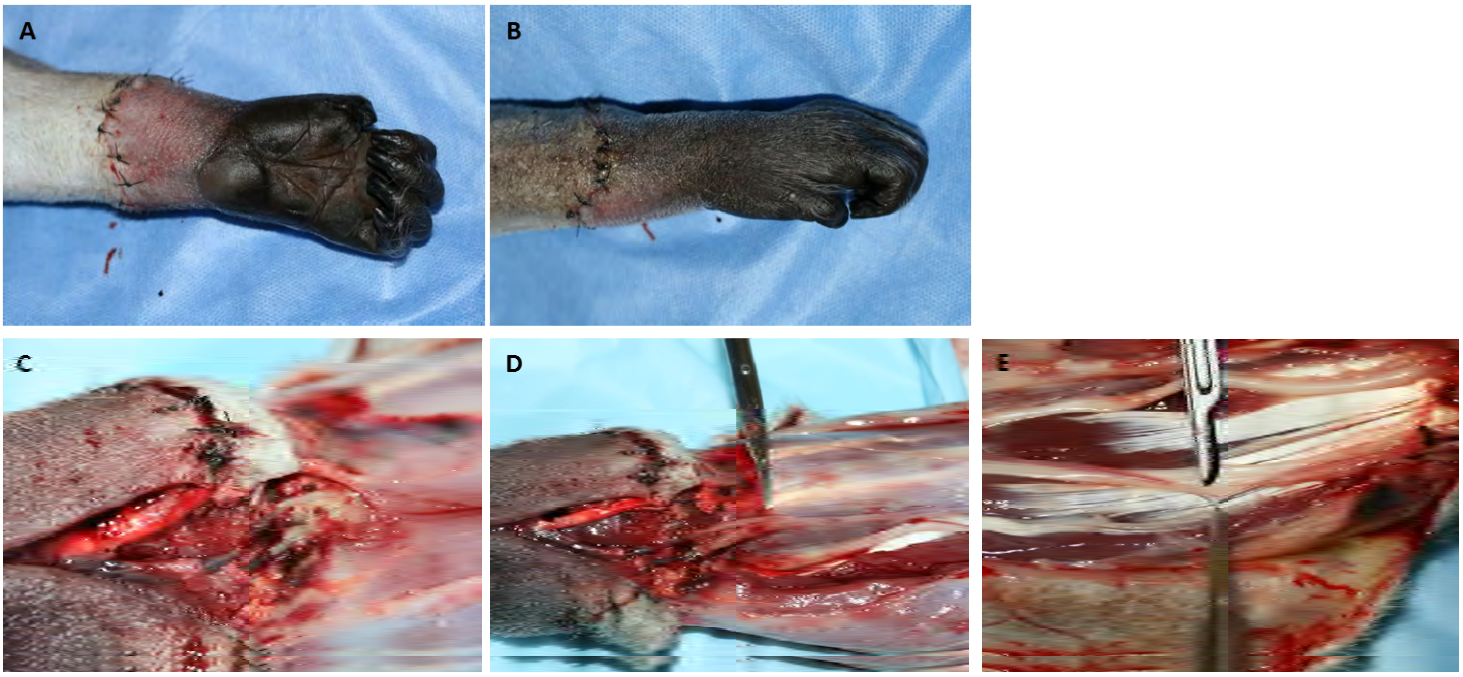

Figure 6. Anatomic analysis of non-human primate forearm vasculature: strategies to improve transplantation success ratio. *Representative images M4413. A) Volar view showing ischemic rubor. B) Radial view. C) Thrombosis extending distal from arterial anastomoses. D) Bifurcation of radial artery. E) Vestigial ulnar artery.*

iv. Discussion

Translation of novel protocols for the immunologic management of patients undergoing restorative transplantation procedures requires the highest level of preclinical scrutiny, and demonstration of the safety and efficacy of proposed protocols in stringent model systems. Non-human primate models are recognized to play a key role in this translational pathway. We have developed a stringent non-human primate upper extremity transplantation model, and during this reporting period have commenced work utilizing this model to the investigation of delayed induction of transplant tolerance; a paradigm with potential application not only to reconstructive transplantation, but to transplantation of solid organs from deceased donors, which has already proven successful in similar non-human primate studies of kidney transplantation (Yamada Y et al, 2012).

The successful execution of experiments of this type is critically dependent on availability of suitable, high quality experimental animals from reliable vendors. We have established a reliable supply chain with Charles River Laboratories, and expect that development of our screening and selection algorithm will facilitate selection, shipping and quarantine of suitable animals in a timely manner in preparation as we progress through the experiments outlined in the statement of work.

While our award modification request is pending we have proceeded with subsequent tasks, and are making good progress against our approved statement of work. We expect this to continue and do not envisage any problems with completing the described work within the projected time period of this award.

With regard to experimental outcomes during this reporting period, the early loss of hands transplanted to M4313 and M4413 was disappointing, but as described above, thorough review of these cases has highlighted a number of opportunities to optimize our protocol, and one potential revision to the surgical technique used, which cumulatively would be expected to significantly reduce the risk of technical failure without substantively modifying the experimental method employed. We do not expect persisting technical issues in this regard, and while occasional technical failures are to be expected we believe the loss of two successive animals from one group during this reporting period to be anomalous.

While the early onset of irreversible acute rejection in M4213 may at first glance also appear to represent a failure, this is not in fact the case, as an incidence of rejection prior to conditioning and attempted tolerance

induction is to be expected in this model, and indeed is experienced in similar studies for kidney (Yamada Y et al, 2012), combined heart-kidney (J Madsen, personal communication) and lung (J Allen, personal communication) allografts. Further investigation as this project continues will identify whether the incidence of such rejection episodes for VCA will be comparable to these solid organ transplants, and what strategies will be necessary to control rejection and prevent sensitization prior to BMT.

In terms of *in vitro* studies, the characterization of bone marrow procured for delayed transplantation and analysis of this data in light of outcomes in subsequent transplantation studies will be an important quality control measure for the cryopreservation process. Ultimately, it can be hoped that specific populations within donor bone marrow may be demonstrated to play critical roles in tolerance induction, and while not a specific aim of this project, analysis of this type can be hoped to contribute to this effort over the long term.

More acutely, optimization of our techniques for detailed characterization of cutaneous leukocytes for use in non-human primates is a key achievement and a highlight of our work during this reporting period. Previous studies in a porcine model have suggested that local mechanisms within the skin of VCAs may play a critical role in determining the outcome, particularly in the context of mixed chimerism protocols (Leonard et al, 2014). Our preliminary data from animal M4213 demonstrating that the findings of these analyses correlate well with gross and histological evidence of acute rejection is encouraging and it can be hoped that these techniques will yield important mechanistic insights, and perhaps demonstrate diagnostic value, as this project progresses.

KEY RESEARCH ACCOMPLISHMENTS

The following represent key accomplishments of this research during this reporting period:

- *Optimization of techniques for analysis of cutaneous immune system in non-human primates.*
    - These techniques, previously developed for human skin and optimized by our group for research in porcine models of VCA, permit comparative analysis of skin immune responses in multiple research species and in humans, which will facilitate broad translation of findings from this work to clinical application.
- *Correlation of flow cytometric analysis of cutaneous immune system with gross and histologic evidence of acute rejection.*
    - Although preliminary, correlation of gross and histological evidence of rejection with infiltration of dermis and epidermis by recipient-type T cells, the majority of which bear a memory phenotype, is encouraging. This technique permits more comprehensive analysis of the cellular populations mediating rejection than can be achieved by histological analysis, and it can be hoped this will yield important mechanistic insights as this work progresses.

CONCLUSION

The induction of transplant tolerance for reconstructive transplantation would be of considerable benefit to civilian victims of disabling and disfiguring tissue loss, and of significant importance to military victims of upper extremity and/or craniofacial trauma. Currently, the necessity of life-long immunosuppression and regular medical monitoring would prevent recipients of restorative transplants (such as hand or face transplant) from returning to active duty, but a safe and effective protocol for induction of transplant tolerance holds the potential to fundamentally change this paradigm.

Introduction of novel protocols of this type to clinical practice clearly requires the highest degree of rigor during pre-clinical testing prior to translation to clinical trial. Consistent with this, research in large animal models is challenging, and unsurprisingly we have faced a number of challenges during this reporting period. However, despite this, progress toward our aims has been steady, the challenges met have been carefully

analyzed and corrective action plans determined. Furthermore, improvements in our ability to isolate and analyze immunologically active cells from small volume skin biopsies is an important development which can be expected to facilitate significant insights into the mechanisms operational in VCA acceptance under immunosuppression, rejection and tolerance, as this project continues.

Overall, despite the obvious challenges encountered, we are encouraged by our overall progress during this reporting period, and expect to proceed apace during year two of this award. We expect that our analysis of the technical failures encountered in two animals, and the lessons learned from this analysis, will facilitate progress in future experiments and that establishment of a reliable procurement pathway will ensure ready availability of optimal experimental animals for this work.

PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

1. Lay Press:

Nothing to report

2. Peer-Reviewed Scientific Journals:

Work supported by this award, in combination with our preliminary studies presented as preliminary data in the application for this award, has so far resulted in the acceptance of one peer-reviewed manuscript for publication:

Leonard DA, Harrison P, Albritton A, Shanmugarajah K, Mastroianni M, Lofgren S, Winter J, Kurtz JM, Cetrulo CL Jr. Upper Extremity Transplantation in Non-Human Primates: An Orthotopic Model for Translational Research. *Journal of Visualized Experiments.* (In Press)

3. Invited Articles:

Nothing to report.

4. Abstracts:

Leonard DA, Mallard C, Shanmugarajah K, Mastroianni M, Albritton A, Powell H, Kurtz JM, Cetrulo CL Jr. Development of an Orthotopic Upper-Extremity Vascularized Allotransplant Model in Non-Human Primates. Poster Presentation. Harvard Medical School Surgical Research Symposium. Boston, Massachusetts. May 2014.

Leonard DA, Mallard C, Shanmugarajah K, Mastroianni M, Albritton A, Powell H, Kurtz JM, Cetrulo CL Jr. Development of an Orthotopic Upper-Extremity Vascularized Allotransplant Model in Non-Human Primates. *Oral Presentation. New England Society of Plastic & Reconstructive Surgeons*. Sebasco, Maine. June 2014.

Leonard DA, Mallard C, Shanmugarajah K, Mastroianni M, Albritton A, Powell H, Kurtz JM, Cetrulo CL Jr. Development of an Orthotopic Upper-Extremity Vascularized Allotransplant Model in Non-Human Primates. *Poster Presentation. Military Health System Research Symposium*. Fort Lauderdale, Florida. August 2014.

Leonard DA, Mallard C, Shanmugarajah K, Mastroianni M, Albritton A, Powell H, Kurtz JM, Cetrulo CL Jr. Development of an Orthotopic Upper-Extremity Vascularized Allotransplant Model in Non-Human Primates. *Poster Presentation. American Society for Reconstructive Transplantation Biennial Meeting.* Chicago, Illinois. November 2014.

INVENTIONS, PATENTS AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

1. Development of an orthotopic upper-extremity vascularized allotransplant model in non-human primates.

2. Validation of techniques for isolation and flow cytometric analysis of skin-resident leukocyte populations in non-human primate skin

OTHER ACHIEVEMENTS

Nothing to report.

REFERENCES

Leonard DA, Kurtz JM, Cetrulo CL Jr. Vascularized composite allotransplantation: towards tolerance and the importance of skin specific immunobiology. Curr Opin Organ Transplant 2013;18:645-51

Clark RA, Chong B, Mirchandani N et al. The vast majority of CLA+ T cells are resident in normal skin. J Immunol 2006; 176:4431-4439

Clark RA, Kupper TS. IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. Blood 2007; 109:194-202

Clark RA, Watandabe R, Teague JE et al, Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. Sci Transl Med 2012;4:117ra7

Eberlin KR, Leonard DA, Austen WG et al, The volar forearm fasciocutaneous extension: a strategy to maximize vascular outflow in post-burn injury hand transplantation. Plast. Reconstr. Surg 2014;134:731

Yamada Y, Boskovic S, Aoyama A, et al. Overcoming memory T cell responses for induction of delayed tolerance in nonhuman primates. Am J Transplant 2012;12:330-40

Leonard DA, Kurtz JM, Mallard C et al. Vascularized composite allograft tolerance across MHC barriers in a large animal model. Am J Transplant 2014;14:343-55

APPENDICES

Appendix 1: Award Modification Request

Appendix 2: Modified Statement of Work (Pending Approval by GOR)- available in Excel

MASSACHUSETTS GENERAL HOSPITAL

HARVARD MEDICAL SCHOOL

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February 5, 2014

US Army Medical Research Acquisition Activity
ATTN: Gay Hayden
820 Chandler Street
Fort Detrick, MD 21702-5014
301-619-9883
gay.c.hayden.civ@mail.mil

Re: W81XWH-13-2-0062
Log #: 120034P5
Tolerance in Nonhuman Primates by Delayed Mixed Chimerism
PI: Curtis L. Cetrulo, Jr., M.D., FACS

Dear Ms. Hayden:

We would like to request permission to amend the Statement of Work (SOW) for our project to reflect findings of preliminary experiments, performed with leveraged institutional funding under independent IACUC approval, which were conducted in the interim period between the submission of this grant application and the necessary IACUC/ACURO approvals to begin work on these studies.

As a result of these preliminary experiments, we have established the following: 1) optimization of the conventional immunosuppression doses (tacrolimus, mycophenolate mofetil and methylprednisolone) required to maintain rejection-free survival of vascularized composite allografts (VCAs) prior to delayed conditioning and donor bone marrow transplantation, and 2) identification of the rate of significant complications, necessitating early withdrawal of animals from the experimental protocol, which is slightly higher than estimated at the time of grant preparation.

Considering these key findings, we request approval to eliminate Aim 1 Group 1 (optimize standard immunosuppression (SIS) protocol for upper extremity transplantation in nonhuman primates) from the SOW (see revised version attached). This amendment will avoid unnecessary duplication of work in identifying optimal immunosuppressive doses for this protocol. Secondly, to address our modest underestimation of the rate of significant complications, we request permission to re-distribute the animals from this deleted group (3

donors and 3 recipients) to the remaining experimental groups, to avoid any negative impact on the significance of our results.

In summary, if this request is approved, the results will be prevention of any unnecessary duplication of work (described under Aim 1 Group 1), and the necessary animal allowance due to a slightly higher than predicted rate of complications. Additionally, this change will allow us to offset the longer than expected IACUC and ACURO approval process, while staying either on or ahead of schedule with our planned experiments. Furthermore, this change will not affect our approved budget.

Sincerely,

Curtis L. Cetrulo, Jr. M.D., FACS

meaghan a mackinnon

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Senior Grants Administrator
Partners Research Management
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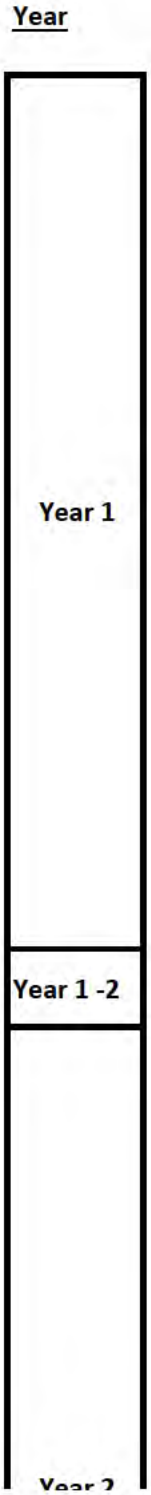
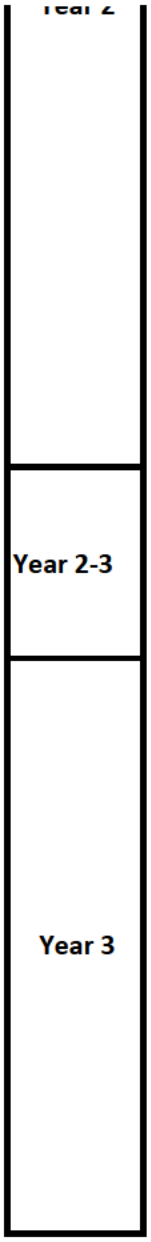
Cc: Partners Research Management
Michael Romanko, Ph.D., RTR Senior Scientific Officer

Project AP1

Title: Tolerance in Non-Human Primates by Delayed Mixed Chimerism
PI: Curtis L. Cetrulo, Jr., MD, FACS, David H. Sachs, MD, Massachusetts General Hospital
Collaborating Investigators: David A. Leonard, MBChB, Josef Kurtz, PhD: TBRC, Massachusetts General Hospital. Zhirui Wang, PhD: RPrEP Core, MGH, DF/HCC

MR120034P5




Year 2-3
Year 3

AIM	TASK
AIM 1. To optimize the delayed tolerance induction protocol for vascularized composite allotransplantation in a non-human primate model.	(1.1) TASK 1. Investigate version 1 delayed tolerance induction protocol (DTIP) for upper extremity transplantation in nonhuman primates. (Months 0-18)
	(1.2) TASK 2. Produce LFA-3-IgG for use in Aim 2/Aim 3 (Months 0-24)
AIM 2. To investigate the effect of T memory cell inhibition and in-vivo T regulatory cell up regulation on the delayed induction of VCA tolerance.	(2.1) TASK 1. Investigate effect of Tmem inhibition on delayed induction of VCA tolerance (Months 12-24)

	(2.2) TASK 2. Investigate effect of Treg up-regulation on delayed induction of VCA tolerance (Months 16-24)
AIM 3. To investigate the effect of combined T memory cell inhibition and T regulatory cell up regulation on the delayed induction of VCA tolerance	**(3.1) TASK 1. Investigate effect of combined Tmem inhibition and Treg up regulation on delayed induction of VCA tolerance (Months 22-36)**

SUBTASK	MONTH(S)	1
(1.1.1) SUBTASK 1. IACUC and ACURO review and approval. (Month 0-4)	Month 0-4	X
(1.1.2) SUBTASK 2. Order and take delivery of first cohort of non-human primates. (Month 5-6)	Month 5-6	
(1.1.3) SUBTASK 3. Orthotopic upper extremity transplants on 4 months SIS (n=4). (Months 6-9)	Month 6-9	
(1.1.4) SUBTASK 4. Delayed tolerance induction protocol, wean immunosuppression. (Months 10-13)	Month 10-13	
(1.1.5) SUBTASK 5. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 10-18)	Month 10-18	
(1.1.6) SUBTASK 6. Summarize preliminary data/progress on DTIP transplants for inclusion in year 1 report(Month 12)	Month 12	
	Month 0-24	X
(2.1.1) SUBTASK 1. Orthotopic upper extremity transplants on 4 months SIS (n=4) (Months 12-15)	Month 12-15	
(2.1.2) SUBTASK 2. Delayed tolerance induction protocol + CTLA4-Ig/LFA-3-IgG. (Months 16-19)	Month 16-19	
(2.1.3) SUBTASK 3. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 16-24)	Month 16-24	
(2.1.4) SUBTASK 4. Summarize and report data on effect of Tmem inhibition on delayed induction of VCA tolerance for year 2 report (month 24)	Month 24	

(2.2.1) SUBTASK 1. Orthotopic upper extremity transplants on 4 months SIS (n=4) (Months 17-20)	Month 17-20
(2.2.2) SUBTASK 2. Delayed tolerance induction protocol + a-IL-6R (Months 20-24)	Month 20-24
(2.2.3) SUBTASK 3. Investigate chimerism, in vitro immune status, VCA survival outcomes following weaning of immunosuppression. (Months 17-24)	Month 17-24
(2.2.4) SUBTASK 4. Summarize and report data on effect of Treg upregulation on delayed induction of VCA tolerance for year 2 report (month 24)	Month 24
(3.1.1) SUBTASK 1. Orthotopic upper extremity transplants on 4 months SIS (n=4) (months 22-25)	Month 22-25
(3.1.2) SUBTASK 2. Heterotopic partial face transplants on 4 months SIS (n=4) (Months 25-28)	Month 25-28
(3.1.3) SUBTASK 3. DTIP with combined Tmem inhibition/Treg upregulation. (Months 26-32)	Month 26-32
(3.1.4) SUBTASK 4. Investigate durability of chimerism, VCA survival, frequency of complications (eg GvHD) and in vitro immune status (Months 26-36)	Month 26-36
(3.1.5) SUBTASK 5. Summarize and report data on effect of combined Tmem inhibition/Treg upregulation on delayed induction of VCA tolerance for year 3 report (month 36)	Month 36
(3.1.6) SUBTASK 6. Complete data analysis, prepare final (year 3) report, prepare manuscripts for submission	Month 36

2	3	4	5	6	7	8	9	10	11
X	X	X							
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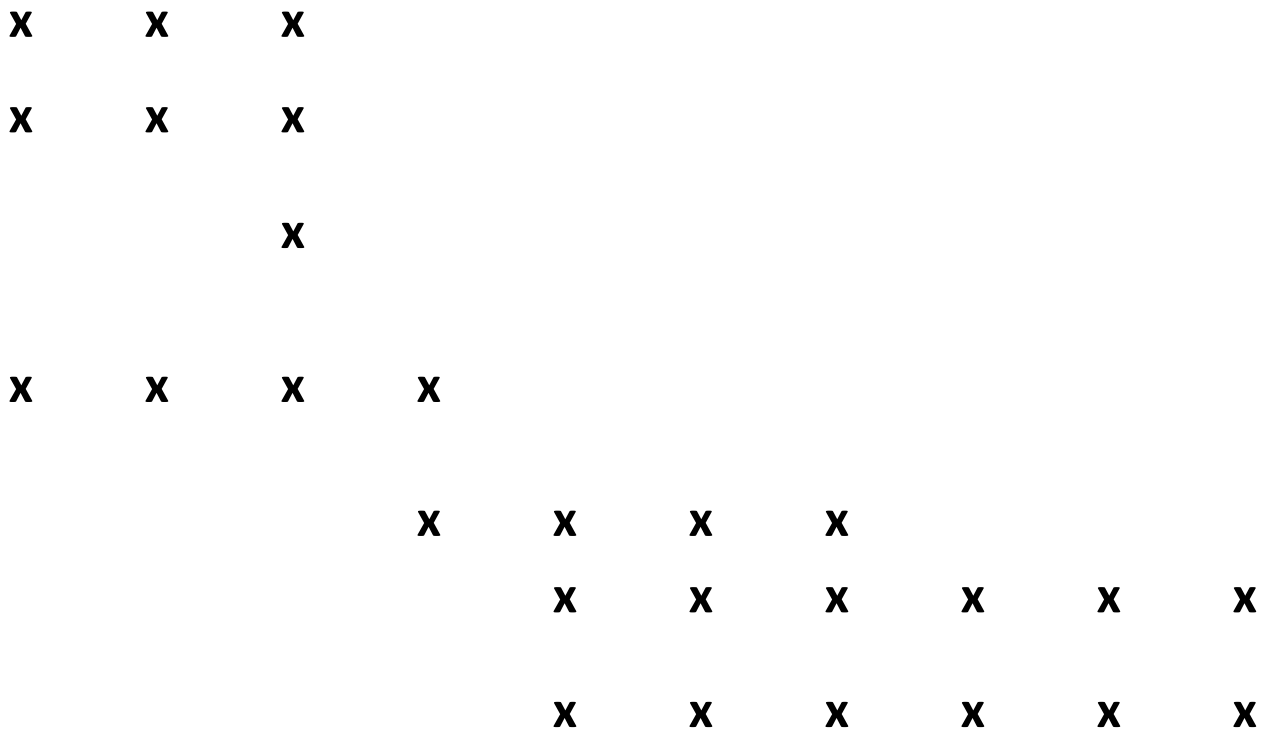

32 **33** **34** **35** **36**